Vascular ATGL-dependent lipolysis protects endothelial function against exogenous lipids overload; involvement of endogenous cPLA$_2$–PGI$_2$ pathway

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Abstract

Adipose triglyceride lipase (ATGL) is involved in lipolysis and displays detrimental pathophysiological role in cardio-metabolic diseases. However, the organo-protective effects of ATGL-induced lipolysis were also suggested. The aim of this work was to characterize the function of lipid droplets (LDs) and ATGL-induced lipolysis in the regulation of endothelial function. ATGL-dependent LDs hydrolysis and cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>)-derived eicosanoids production were studied in the aorta, endothelial and smooth muscle cells exposed to exogenous oleic acid (OA) or arachidonic acid (AA). Functional effects of ATGL-dependent lipolysis was studied in vitro in endothelial barrier integrity assay and in vivo in relation to postprandial endothelial dysfunction.

The formation of LDs was invariably associated with elevated production of endogenous AA-derived prostacyclin (PGI<sub>2</sub>). In the presence of the inhibitor of ATGL or the inhibitor of cytosolic phospholipase A<sub>2</sub>, production of eicosanoids was reduced, with concomitant increase in the number of LDs. OA administration impaired endothelial barrier integrity in vitro that was further impaired if OA was given together with atglistatin. In vivo, olive oil induced postprandial endothelial dysfunction that was significantly deteriorated by ATGL inhibition.

In summary, vascular lipid droplets formation was associated with ATGL- and cPLA<sub>2</sub>-dependent PGI<sub>2</sub> production from endogenous AA. The inhibition of ATGL resulted in an impairment of endothelial barrier function in vitro and deterioration of endothelial function upon exposure to olive oil in vivo. These results demonstrate that vascular ATGL-cPLA<sub>2</sub>-PGI<sub>2</sub>-dependent pathway activated by lipid overload and linked to LDs formation in endothelium and smooth muscle cells has a vasoprotective role and counterbalances detrimental vascular effects of lipid overload.

1. Introduction

Adipose triglyceride lipase (ATGL) has been identified as a key enzyme of mammalian lipolysis, involved in the hydrolytic cleavage of triglycerides into free fatty acids and diacylglycerols. ATGL mainly localizes to TG-rich intracellular lipid droplets, and it is predominantly expressed in adipose tissue. However ATGL was also found to a lesser extent in a variety of other tissues and organs, including kidney, liver, skeletal muscle, immune cells lung and heart [1–7]. Studies with the use of genetic mouse models with tissue-specific overexpression or deletion of ATGL as well as with the use of the recently developed ATGL specific inhibitor (atglistatin) gave unprecedented insight into the pathophysiological role of ATGL.

Quite intriguingly, ATGL-dependent lipolysis was reported to display detrimental or beneficial role, depending on targeted organ or tissue. In the liver, ATGL-mediated lipolysis plays an important function in hepatic lipid homeostasis [1] and ATGL inhibitor, atglistatin substantially reduced high fat diet-induced hepatosteatosis, obesity, liver inflammation and hepatic fibrosis. Moreover, atglistatin effectively diminished the metabolic consequences of obesity such as insulin resistance and non-alcoholic fatty liver disease (NAFLD) in mice fed a high-fat diet [2].
In relation to cardiac pathology, number of reports demonstrated beneficial effects of atglistatin. Botterman et al demonstrated that inhibiting lipolysis with atglistatin was able to improve cardiac function after myocardial infarction [4]. Inhibition of ATGL afforded also the cardioprotective effect on catecholamine-induced cardiac damage [5] and protected against heart failure induced by pressure-overload [6, 7].

In other organs, targeting ATGL using atglistatin had a wide spectrum of therapeutic effects. For example, in severe burn injuries resulting in hypermetabolic response together with hyperlipidemia and fatty liver development, atglistatin protected against the development of fatty liver and post-burn injury[3]. Furthermore, ATGL-mediated lipid hydrolysis had an important role in bronchial regeneration. Deletion of the gene encoding ATGL induced substantial triglyceride accumulation, decreased mitochondrial numbers, and decreased mitochondrial respiration in club cells [8]. Finally, loss of the Atgl gene induced the development of spontaneous pulmonary neoplasia, which progressed to adenocarcinoma [9].

Despite the progress in the studies related to the role of ATGL in various organs and diseases, still little is known about the role of ATGL-dependent lipolysis in vascular wall linked to LDs formation. Furthermore, it is not clear whether the vascular ATGL- pathway has beneficial or detrimental role in the maintenance of endothelial function.

Initially, LDs formation in vascular wall was related to pathophysiology of atherosclerosis [10, 11]. Recently, however, in an elegant study by Kuo et al, it was demonstrated that lipid droplet metabolism protected endothelial cells from lipotoxicity and provided fatty acids for mitochondrial function and transport to adjacent cells [12]. In a number of recent reports from our group, the biochemical content of LDs in endothelial cells was analyzed using Raman spectroscopy [13, 14] and it was demonstrated that endothelial inflammation in cultured endothelial cells or in the isolated murine aorta was invariably associated with the formation of LDs suggesting that their formation is an integral component of vascular inflammation [15, 16]. Noteworthy, the role of LDs in leukocytes in the generation of eicosanoids is well accepted [17, 18] as well as their modulatory role in inflammatory and immune responses of leukocytes [19]. In fact, it is well-known that inflammatory response in leukocytes can be amplified not only by the enzymatic decomposition of membrane phospholipids but also by the hydrolysis of AA-rich triglycerides stored in LDs in some cells.

Vascular wall homeostasis and inflammation is highly regulated by eicosanoids [17, 19–23]. Although it is already known that LDs formation may be linked to PGI\textsubscript{2} generation in response to a wide range of stimuli [24] yet, the functional consequences of LDs formation and eicosanoids generation linked to LDs formation in terms of endothelial function have not been elucidated.

The aim of this work was to investigate the functional role of LDs in the regulated formation of AA-derived eicosanoids via ATGL/cPLA\textsubscript{2} pathway in the vascular wall. Firstly we characterized in details ATGL-dependent lipid droplets formation and hydrolysis, cytosolic phospholipase A\textsubscript{2}-dependent eicosanoids production in the aorta, endothelial and smooth muscle cells exposed to exogenous oleic
acid (OA) and arachidonic acid (AA), using fluorescence and Raman imaging as well as LC/MS/MS-based eicosanoids analysis. Then, we assessed the functional role of ATGL-dependent lipolysis offsetting postprandial endothelial dysfunction \textit{in vivo} and in the preservation of endothelial barrier integrity \textit{in vitro}.

2. Materials and methods

2.1. Animals and aorta isolation

C57/BL/6 mice (wild type) at the age of 8–12 weeks were kept under controlled conditions (22–24°C, 55% humidity, 12 h day/night rhythm with free access to food and water until the day of the experiment). All experimental procedures involving animals were conducted according to the Guidelines for Animal Care and Treatment of the European Communities and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85−23, revised 1996). All procedures were approved by second Local Ethical Committee on Animal Experiments. Mice were euthanized by an intraperitoneal injection of a mixture consisting of ketamine and xylazine (100 mg ketamine/10 mg xylazine/kg body weight). The chest was opened and the thoracic aorta was removed and transferred into Krebs–Henseleit buffer. Subsequently, the aorta was cleaned from surrounding adipose tissue and transferred into medium (minimal essential medium (MEM, Sigma Aldrich) with the addition of 1% MEM vitamins (Sigma Aldrich), 1% antibiotics (penicillin 10,000 U/mL and streptomycin 10,000 µg/mL, ThermoFisher Scientific) and 1% non-essential amino acids, Sigma Aldrich). The aorta was incubated with oleic acid (Cayman Chemical) at a concentration of 500 µM or 1mM in the presence or absence of lipolysis inhibitor, atglistatin (50 µM, Cayman Chemical) and phospholipase A$_2$ inhibitor, arachidonoyl trifluoromethyl ketone (AACOCF3, 10 µM, Abcam) for 4 hrs or 24 hrs. Aortic samples were incubated at 37°C and 5% CO$_2$. For immunofluorescence staining and Raman imaging of aorta \textit{en face}, the resected and split-open arteries were tightly glued to the Cell-Tak® (Corning)-coated microscopic glasses and calcium fluoride surface, respectively. Subsequently, the tissues were preserved by a 15-min soak in 4% paraformaldehyde (fluorescence imaging) or 10-min soak in 4% buffered formalin (Raman imaging).

1.2. Cell culture

Human aorta endothelial cells line (HAEC) was obtained from Lonza (Basel, Switzerland) Vascular smooth muscle cell line (MOVAS) was purchased from American Type Culture Collection (ATCC, Rockville, Maryland, MD, USA). HAECs were maintained in supplemented endothelial growth medium EGM-2 (Lonza, Basel, Switzerland, MOVAS cells were cultured in DMEM supplemented 10% (v/v) FBS (both from Gibco, Scotland, UK). Cultured were maintained at 37°C in a humidified atmosphere of 5% CO2-95% air. When cells achieved 90% confluence the medium was changed and cells were treated with 100 µM oleic acid (Cayman Chemical) or arachidonic acid (25 µM, Cayman Chemical) in the presence or absence of lipolysis inhibitor, atglistatin (50 µM) or phospholipase A$_2$ inhibitor (AACOCF3, 10 µM, Abcam) for 4 hrs or 24 hrs. Oleic acid (Cayman Chemical) for the experiments was freshly saponified using 100
mM NaOH and BSA-conjugated (10% in DPBS, low endotoxin, fatty acid free, suitable for cell culture, sterile-filtered; Sigma Aldrich). After 4 hrs or 24 hrs of incubation cells were fixed for 4 min with 4% paraformaldehyde. Fixed cells were washed 3 times with PBS and stored in PBS at 4°C until the execution of the measurements.

1.3. Immunostaining of the aorta en face

**LDs and CD31 detection**

Thoracic aorta isolated from 8–12 weeks old C57BL/6 mice was dissected and cut longitudinally. Aortic samples were pinned down with EC facing upward, washed with PBS 3 times and fixed with 4% paraformaldehyde in PBS for 15 mins. Fixed samples were further blocked with TNB blocking buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, and 0.5% (w/v) and blocking reagent (PerkinElmer) for 3–4 hrs, and then incubated with CD31 antibody (Abcam, 1:50) diluted in TNB blocking buffer overnight at 4°C. As secondary antibody, Alexa Fluor 647 nm goat-anti-rabbit (Jackson Immuno Research; 3:600) was used at room temperature for 3 hrs. BODIPY 493/503 (Invitrogen) diluted in PBS at the final concentration of 0.1 mg/ml was applied for 1 hr to delineate LDs, and Hoechst 33258 (Sigma Aldrich; 1:1000) was used to highlight nuclei. Samples were visualized on CQ1 Confocal Quantitative Image Cytometer (Yokogawa, Japan) at 40× magnification.

1.4. Raman imaging of aorta en face

Raman imaging was carried out using a WITec Confocal Raman Imaging system (WITec alpha300, Ulm, Germany) supplied with a UHTS 300 spectrograph (600 grooves·mm$^{-1}$ grating, resolution of 3 cm$^{-1}$) and a CCD detector (Andor, DU401A-BV-352). The air-cooled solid state laser with the excitation wavelength of 532 nm was coupled to the microscope via an optical fiber with a diameter of 50 µm. Raman spectra of tissues were collected with the application of a 63× water immersive objective (Zeiss Fluor, NA = 1.0), using maximum laser power at the sample position (ca. 30 mW) and 0.4 s exposure time per spectrum. The nominal minimal lateral and depth resolution for our setup is 0.32 and 0.53 µm, respectively, and sampling density of 0.38–0.50 and 0.5–1.0 µm in x/y and z direction, respectively, was used. Depth profiling of the tissue was obtained by multiple imaging of the same line in several layers of the sample. The distribution images collected at different depths present the relative intensity of a studied component in the tissue. Data matrices were analysed using a WITec Project software (background subtraction using a polynomial of degree 2 and the automatic removal of cosmic rays). The analysis of the spectra was supported by a Cluster Analysis (CA) (K-means, Manhattan distance, WITec Project Plus). This approach enabled data grouping into classes and extraction of average spectra reflecting the major compartments inside tissues. For study of heterogeneity of LDs observed in in situ endothelial cells within isolated blood vessels treated with OA or dOA, the single Raman spectra were extracted from the centre of each LD, and then averaged. The OPUS 7.2 program was used for calculations of the integral intensity of the bands at ca. 2940 (reflecting the overall content of lipids), 1745 (esters), 1660 (level of unsaturation), 1445 (level of unsaturation), 702 (cholesterols) cm$^{-1}$ in the 2815–3033, 1733–1767, 1563–1712, 1394–1505 and 695–709 cm$^{-1}$ spectral ranges, respectively. Integration was performed using method D, OPUS 7.2: the
integral was defined by the wavenumber limits and the horizontal baseline determined by a chosen baseline point.

1.5. LC-MS/MS Eicosanoid Analysis

Selected eicosanoids were quantified in medium collected after aorta incubation and cell culturing using a LC-MS/MS-based method with the application of an already published methodology [25]. In short, each sample was spiked with a mixture of internal standards and gently mixed. Next, samples were cleaned up using liquid-liquid extraction by the addition of acidified ethyl acetate. After vigorous shaking and centrifugation, the organic layer was transferred to a fresh tube, and evaporated to dryness under a nitrogen stream (37°C). The dry residue was dissolved in ethanol and after centrifugation clean samples were injected into LC-MS/MS system comprising of a Nexera UFLC (Shimadzu, Kyoto, Japan) ultrafast liquid chromatograph combined with a QTrap 5500 (Sciex, Framingham, Massachusetts, USA) triple quadrupole mass spectrometer. The detailed conditions of chromatographic separation and mass spectrometric detection as well as sample preparation procedure were described in our previous work [25].

The lipid extract from HAEC was subjected to AA and AA-d8 LC-MS/MS analysis using an Ultimate 3000 UHPLC (Dionex; Sunnyvale, California, USA) liquid chromatograph and TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific). The best chromatographic separation was achieved on an Acquity UPLC BEH C18 (3.0 × 100 mm, 1.7 µm, Waters, Milford, Massachusetts, USA) analytical column. The mobile phases consisted of ACN (A) and H2O (B) were delivered at a flow rate of 550 µL/min employing isocratic elution mode (A:82% and B:18%). The mass spectrometric detection was carried out in negative ionization applying Selected Reaction Monitoring (SRM) mode including the most abundant and specific Q1→Q3 ion transitions: 303.1→259.3, 311.3→267.2 and 314.2→270.4 for AA, AAd8 and AA-d11 (internal standard), respectively. The sample clean up protocol was the same as applied for eicosanoid determination.

1.6. Assessment of endothelial function \textit{in vivo} by MRI

In experiments \textit{in vivo}, the effect of atglistatin (50 uM) was studying on olive oil (10 ml/kg bw)- induced postprandial endothelial dysfunction. The dose of olive oil (10 mg/ml bw, Sigma Aldrich) was chosen based on the plasma triglycerides measurements curve to ensure the postprandial effect and vascular LDs formation. MRI experiments were performed using a 9.4T scanner (BioSpec 4/20 USR, Bruker, BioSpin GmbH, Germany), as described previously [26]. Mice were anesthetized using isoflurane (Aerrane, Baxter Sp. z o. o., Warszawa, Poland, 1.7 ol. %) in an oxygen and air (1:2) mixture. Body temperature was maintained at 37°C using circulating warm water. ECG, expiration and body temperature were monitored using a Model 025 Monitoring and Gating System (SA Inc., Stony Brook, NY, United States). Endothelium-dependent vasodilation was assessed \textit{in vivo} based on the aorta response induced by acetylcholine (Ach) administration, as described previously [27, 28]. Response to injection of Ach (Sigma–Aldrich, Poznan, Poland: 50 µl, 16.6 mg/kg, \textit{i.p.}), was analyzed in the lower part of the thoracic aorta (ThA) and the abdominal aorta (AbA). Vasomotor response was examined based on time-resolved 3D images of the vessels prior to and 25 min after intraperitoneal Ach administration (time was determined experimentally.
in our previous study) [26]. Images were acquired using the cine IntraGateTM FLASH 3D sequence, reconstructed with the IntraGate 1.2.b.2 macro (Bruker). End-diastolic volumes of vessels were analyzed using ImageJ software 1.46r (NIH, Bethesda, MD, United States) and scripts written in Matlab (MathWorks, Natick, MA, United States). Imaging parameters included the following: repetition time (TR) – 6.4 ms, echo time (TE) – 1.4 ms, field of view (FOV) – 30 mm × 30 mm × 5 mm, matrix size – 256 × 256 × 30, flip angle (FA) – 30°, and number of accumulations (NA) – 15, reconstructed to seven cardiac frames. Total scan time was 10 min.

1.7. Endothelium permeability measurement in vitro

The response of the barrier formed by human aortic endothelial cells (HAEC) to an oleic acid (OA, 100 µM) in the presence or absence of atglistatin (50 µM) was assessed in real-time in a fully standardized manner by continuously recording changes in electrical resistance with an ECIS system [27], using 96W10E + electrode chamber arrays and an ECIS Z-Theta system (Applied Biophysics), along with the associated software v.1.2.126 PC. Immediately after HAEC seeding, resistance (Ω) measurements were initiated. Once stable resistance was reached, HAEC cells were subjected to treatment with OA alone or in combination with ATGL for 24 hrs and the endothelial-barrier resistance was measured in real-time at multiple frequency modes (ranges between 250 and 64,000 Hz). The experiment was performed in a humidified 5% CO2 incubator at 37°C and was repeated three times in seven technical replicates [29].

1.8. Statistical Analysis

All of the data obtained are presented as mean and standard deviation (SD) or in case of the lack of normal distribution as median with interquartile range. Statistical tests were done using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, United States) software. Non-parametric test (Kruskal–Wallis test followed by Dunn’s post hoc test) or parametric test (one-way ANOVA followed by Tukey’s or Bonferroni’s post hoc test) were performed. Statistical significance was defined as $p < 0.05$.

3. Results

2.1. Exogenous AA induces LDs formation and ATGL and cPLA$_2$-dependent PGI$_2$ release generated from endogenous AA in endothelial cells and in smooth muscle cells

Incubation of endothelial cells with exogenous AA resulted in LDs formation that was significant 4 hrs after AA administration and further slightly increased after 24 hrs of AA incubation (Fig. 1A). AA-induced LDs formation was associated with elevated production of PGI$_2$ as evidenced by increased 6-keto-PGF$_{1\alpha}$ concentration 4 hrs after AA administration, that further slightly increased after 24 hrs of AA incubation (Fig. 1B).

LDs formation was also associated with a considerable activation of PGF$_{2\alpha}$ production that increased 4-24 hrs after AA administration with a similar kinetics as that of 6-keto-PGF$_{1\alpha}$ (Fig. 1C). Interestingly, AA induced also an increase in PGD$_2$ and PGE$_2$ production (Fig. 1D, 1E). In the presence of ATGL inhibitor,
atglistatin (50 µM), the number of AA-induced LDs in endothelial cell was not significantly altered, but the concentration of 6-ketoPGF$_{1\alpha}$ (Fig. 1A, 1B) was significantly lowered. Similarly, AACOCF3, the inhibitor of cytosolic phospholipase A$_2$ inhibited the production of PGI$_2$ and lowered 6-keto-PGF$_{1\alpha}$ level (Fig. 1B). However, in contrast to the effects of atglistatin, in the presence of AACOCF3, the number of AA-induced LDs was increased (Fig. 1A).

Effects of atglistatin and AACOCF3 in smooth muscle cells were similar as in endothelial cells. AA-induced increase in 6-keto-PGF$_{1\alpha}$ concentration was inhibited. Furthermore, the number of AA-induced LDs was increased 24 hrs after AA administration (Fig. 1.1A, B, supplemental materials).

Interestingly, there was a divergent effects of atglistatin and AACOCF3, on other eicosanoids released by AA either in endothelial cell (Fig. 1C-E) or in smooth muscle cells (Fig. 1,1C-D, supplemental materials). While these inhibitors inhibited PGF$_{2\alpha}$ formation to similar extend as 6-keto-PGF$_{1\alpha}$, their effects on PGD$_2$ and PGE$_2$ formation, was absent or minimal.

To confirm whether eicosanoids are generated from exogenous or endogenous AA, exogenous deuterated AA (AAd8) was used. As shown in Fig. 2, AAd8-induced a significant increase in 6-keto-PGF$_{1\alpha}$ concentration comparable to the effect induced by non-deuterated AA. Moreover, 6-keto-PGF$_{1\alpha}$ release by AAd8 was inhibited by AACOCF3, SC-560 (Cayman Chemical) and DuP-697 (Cayman Chemical) (inhibitors specific for cPLA$_2$, COX-1 and COX-2, respectively) suggesting that exogenous AA induced PGI$_2$ synthesis from endogenous AA that was mediated by cPLA$_2$/COX1/COX2 pathway (Fig2. C).

### 2.2. Exogenous OA induces PGI$_2$ release generated from endogenous AA in endothelial cells, in smooth muscle cells and in isolated murine aorta

Similarly, to exogenous AA, oleic acid (OA) induced LDs formation and PGI$_2$ release in endothelial cells and vascular smooth muscle cells.

As shown in Fig. 3, incubation of endothelial cells with deuterated oleic acid (OAd34) increased the number of LDs. The effect was already seen 1 hr after OAd34 addition and remained at similar level 2,3,6, and 24 hrs after OA administration to endothelial cells (Fig. 3A).

In smooth muscle cells number of LDs induced by OAd34, was significantly higher after 24h as compared to shorter incubation times (Fig. 3B).

Parallel to the increase in LDs formation, the 6-keto-PGF$_{1\alpha}$ concentration was elevated in either endothelial cells or in smooth muscle cells. However, a significant increase in 6-keto-PGF$_{1\alpha}$ concentration induced by OAd34 in HAEC or MOVAS, was delayed as compared with rapid LD formation and was most substantially increased 24 hrs after OA administration (Fig. 3C, 3D). Interestingly, the concentration of 6-keto-PGF$_{1\alpha}$ released by OAd34 was higher in MOVAS as compared with HAEC (Fig. 3D).
To further confirm that exogenous OA resulted in LDs formation that was associated with PGI$_2$ release biochemical composition of LDs induced by OA and 6-keto-PGF$_{1\alpha}$ released was studied in isolated aorta en face.

As shown in Fig. 4, OA induced LDs formation in endothelial layer as well as in smooth muscle cells in the isolated aorta as evidenced by fluorescence staining. The number of LDs in endothelium was significantly higher 24 hrs after OA administration (Fig. 4A).

Interestingly, in smooth muscle the increased LDs number was observed already 6 hrs after OA administration and remained at high level also 24 hrs after OA administration (Fig. 4B).

In the presence of atglistatin, but not AACOCF3, the OA-induced LDs formation in aorta was significantly increased (Fig. 4C). Raman spectroscopy confirmed the presence of LDs in endothelium and in deeper layers of the vessel wall after incubation with OAd34, and identified LDs rich in exogenous oleic acid (OAd34) uptaken from medium and LDs containing more unsaturated lipids then OAd34, most likely AA (Fig. 4E, 4F).

Similarly to the experimental system of isolated endothelial cells (Fig. 3A, 3C) and smooth muscle cells (Fig. 3B, 3D), OA-induced LDs in isolated murine aorta was associated with a time-dependent PGI$_2$ production as evidenced by 6-keto-PGF$_{1\alpha}$ release that was most pronounced 24 hrs after OA administration (Fig.5A). Again, atglistatin and AACOCF3 inhibited OA-induced PGI$_2$ production as well as other prostanoids release (6-keto-PGF$_{1\alpha}$, PGD$_2$, PGE$_2$) indicating that ATGL and cPLA$_2$ are required for LDs metabolism in vascular wall and mediate the production of vascular PGI$_2$ in response to exogenous OA (Fig. 5E-G).

2.3. ATGL-dependent lipolysis regulates endothelial barrier function and postprandial endothelial effects

Functional role of ATGL-dependent lipolysis induced by OA, was analyzed by studying effects of atglistatin (50 µM) on OA (100 uM)-induced changes in endothelial barrier permeability in an in vitro assay and on olive oil (10 mL/kg bw) induced postprandial endothelial dysfunction in vivo.

As shown in Fig. 6, OA alone did not induced significant variation in the cells’ monolayer resistance as assessed by ECIS, however, the combination of OA with atglistatin caused a decrease in the resistance of the endothelial cells, indicating impairment of the endothelial barrier integrity (Fig. 6).

In in vivo experiments the dose of olive oil (10 mg/ml bw) was chosen based on plasma triglycerides measurements curve Plasma TG levels peak was obtained 360 min after olive oil administration (Fig. 7A), whereas LDs were detected 180 min after olive oil administration and were not been visible at later time points. LDs were detected via fluorescence and Raman imaging (Fig. 7B, 7C, 7D), and Raman spectrum of LDs indicated a higher lipid unsaturation than the administered olive oil as the ratio of the bands 1660 to 1446 cm$^{-1}$, and 1266 to 1305 cm$^{-1}$ was higher for the LDs spectrum than for olive oil. Interestingly, the fatty acid profile measured in isolated aorta wall by chromatography-mass spectrometry showed
changes after olive oil administration in unsaturated fatty acids: oleic (18:1) and palmitoleic acid (16:1) as well as linoleic acid (18:2, converted from oleic acid) that were higher after 9 hrs as compared to 6 hrs after olive oil suggesting lipolysis. Furthermore, fatty acids: 18:1, 16:1 content was lower after olive oil and atglistatin treatment as compared to olive oil alone compatible with the inhibition of lipolysis. However, in the case of arachidonic acid, the effects induced by olive oil alone or together with atglistatin was opposite as compared to effects for FA 18:1 and 16:1 (data not shown).

On the functional level, as shown in Fig. 7E-H, olive oil administration induced endothelial dysfunction as evidenced by impaired Ach-induced endothelium-dependent vasodilation in the thoracic and in the abdominal aorta in C57Bl/6 mice 6 hrs after olive oil administration. In the presence of atglistatin (200 µmol/kg bw), Ach-induced response was further deteriorated (Fig. 7E, 7F) suggesting that postprandial endothelial dysfunction was aggravated by ATGL blockade in vivo. In contrast, sodium nitroprusside (SNP)-induced endothelium-independent response in thoracic and abdominal aorta was not affected by olive oil gavage and by atglistatin (Fig. 7G, 7H) which confirms specific protective effects of ATGL-dependent lipolysis on endothelial function in postprandial phase after olive oil administration.

4. Discussion

In the present work we demonstrated that ATGL-dependent lipolysis maintains vascular homeostasis by cPLA2-dependent eicosanoids production from endogenous AA. In particular, we showed the inhibition of ATGL resulted in the deterioration of endothelial function upon exposure to olive oil in vivo as well as in the impairment of endothelial barrier function exposed to oleic acid in vitro. Our results indicated that lipid overload of vascular wall, or endothelial/smooth muscle cells alone resulted in LDs formation that was tightly linked with the activation of endogenous ATGL/cPLA2/AA/PGI2 pathway to protect against detrimental effects of lipid overload on endothelial function. Accordingly, the inhibition of ATGL, suggested to be a therapeutic target in liver inflammation and NAFLD [2] myocardial injury [4, 5] or heart failure [6, 7] may lead to detrimental effects on endothelial function. Similarly impaired vascular ATGL activity induced by constitutive or genetic factors [30] may contribute to the deterioration in endothelial function and its clinical consequences.

It is well known that endothelium and smooth muscle cells are able to produce PGI2, and vascular PGI2 has vasoprotective, anti-thrombotic or anti-proliferative effects linked to cPLA2/COX1/COX2 pathway. However, little was known on the relationship of ATGL/cPLA2/PGI2 pathway. Although previous reports demonstrated that LDs formation in endothelium was linked to PGI2 generation [15, 31, 32] the functional consequences and pathway involved was not investigated. Here, we clearly demonstrated that exogenous OA or AA induced the formation of LDs and the LDs formation in endothelial cells, smooth cells or in the isolated aorta was associated with subsequent hydrolysis of AA-rich triglycerides stored in LDs and elevated production of endogenous AA-derived PGI2 and other eicosanoids. Furthermore, in our hands, the inhibition of cPLA2 or in some experimental settings, the inhibition of ATGL was associated with the increased number of LDs, suggesting that the formation of vascular LDs is dynamically linked with
ATGL/cPLA_2-dependent lipolysis and generation of eicosanoids. The major product of ATGL/cPLA_2-dependent lipolysis was PGI_2 but other eicosanoids were also generated. Interestingly, while PGI_2 was inhibited by atglistatin in endothelial cells and smooth muscle cells, this effect was not observed in relation to PGD_2 and PGE_2 suggesting that LDs formation in endothelium or smooth muscle cells, is linked to the major vascular metabolite of arachidonic acid pathway PGI_2 rather than to PGD_2 and PGE_2.

In the recent studies the relationship between increased PGI_2 production and LDs formation was reported for the high concentration of exogenous free fatty acids: oleic [12] or arachidonic [33]. Increased generation of PGI_2 concomitant to LDs formation in vascular wall in response to OA and other pro-inflammatory factors was also reported recently by our group [24]. In the present work, however we demonstrated that LDs formation was linked in the time-dependent matter with subsequent ATGL/cPLA_2-dependent lipolysis and the release of PGI_2 that was generated from endogenous AA. This scenario pertained to endothelial cells, smooth cells in culture and intact isolated aorta.

In the present work, we used couple of experimental approaches to provide evidence that the release of PGI_2 originated from endogenous sources of AA, independently whether AA or OA was used as a stimulus for LDs formation. Firstly, to confirm whether eicosanoids are generated from endogenous AA, exogenous deuterated AA (AAd8) or deuterated OA (OAd34) was used. We demonstrated that AA8 induced a significant increase in 6-keto-PGF_1α concentration comparable to the effect induced by non-deuterated AA. Moreover, we demonstrated that 6-keto-PGF_1α release by AA8 was inhibited by AACOCF3, SC-560 and DuP-679 (inhibitors specific for cPLA_2, COX-1 and COX-2, respectively) suggesting that exogenous AA induced PGI_2 synthesis from endogenous AA that was mediated by cPLA_2/COX1/COX2 pathway.

Furthermore, similarly, to exogenous AA, also OAd34 induced LDs formation and cPLA_2-dependent PGI_2 release generated from endogenous AA in endothelial cells, vascular smooth muscle cells as well as isolated vessel. Importantly, Raman imaging used in our study further pointed out to a possible presence of endogenous AA in LDs in isolated aorta formed in the response to dOA. In fact, LDs contained unsaturated lipids that could be attributed to AA, but definitely not to dOA [34].

The biochemical content of endothelial LDs was previously, studied by our group in the cultured endothelial cells (HAEC) and in the isolated aorta, upon incubation with OAd34. As the major component OA was revealed (80–90%) with AA contribution (8.7–19.4%) [13]. Altogether, based on these results we may suppose that the formation of LDs even in response to OA and despite an excess of OA is related to the endogenous AA-induced eicosanoids biosynthesis in vascular wall.

The important aspect of this work was to demonstrate the vasoprotective activity of ATGL-dependent lipolysis in the context of postprandial endothelial dysfunction induced by olive oil administration, a well-known model of postprandial response. The novelty of our approach was to took advantage of our unique approach using MRI [26] to study endothelial function in vivo in postprandial phase and to show the vasoprotective functional role of ATGL-dependent lipolysis in the regulation of endothelial function in...
the postprandial phase. Our results clearly showed that inhibition of ATGL resulted in deterioration of endothelial function upon exposure to lipids, pointing out that ATGL-dependent lipolysis affords vasoprotective action offsetting detrimental vascular effects of lipid overload during postprandial hyperlipidemia. Similarly, in *in vitro* assay, lipid overload in the presence of ATGL inhibition resulted in impaired endothelial barrier function further supporting ATGL-dependent lipolysis in maintaining endothelial function in the context of lipid overload.

Previous studies have highlighted the role of ATGL in lipid metabolism in regulating various aspects of fatty acid uptake and LD size [35]. Systemic knockout of ATGL resulted in a distinct murine phenotype that was characterized by cardiac steatosis and severe heart failure as well as impaired endothelial NO synthase expression and activity [36]. LDs formation was shown to protect endothelial cells from lipotoxic stress, providing a source of fatty acids for adjacent cells in the vessel wall or tissues [12]. Finally, it was also shown that in vascular endothelial cells, the efficiency of stimulus-induced AA release and prostacyclin secretion was dependent on ATGL. However, the role of ATGL-induced lipolysis in the regulation of endothelial function during postprandial hyperlipidemia and the mechanistic insight linking LDs formation with the activation of endogenous ATGL/cPLA2/AA/PGI$_2$ pathway shed a novel light into the pathophysiological relevance of the formation and the vasoprotective role of vascular LDs [37].

It is known since many years that elevated nonfasting triglyceride levels were associated with the increased risk of cardiovascular events [38]. Furthermore, there is an association between postprandial dysmetabolism and both coronary artery disease and cardiac events [39, 40]. Accumulation of triglyceride-rich lipoproteins promotes the development of atherosclerosis [41] and a delayed clearance of postprandial lipoproteins from the plasma was suggested to play a role in the etiology of premature coronary atherosclerosis [42, 43]. Given that humans spend the majority of their day in a postprandial (fed) state, with a continual fluctuation in the degree of lipemia throughout the day [44] the effects of postprandial state on endothelial function may play an important determining role in the development of atherosclerosis. A large number of studies have shown that endothelial function assessed as flow-mediated vasodilation (FMD) was affected by postprandial lipemia with various mechanisms proposed including oxidative stress, inflammatory burden and others [45].

In our work we set the animal model of postprandial endothelial dysfunction to show that parallel to maximal serum TG concentration the endothelial dysfunction developed as evidenced by impaired Ach-induced vasodilation in thoracic and abdominal aorta, with concomitant presence of LDs in the early but not late phase of postprandial response confirming the dynamics of LDs formation and lipolysis. Importantly, inhibition of lipolysis impaired the endothelial function while NO-independent vasodilation induced by SNP was not changed. In addition as one of the phenotypic feature of endothelial dysfunction is linked to changes in endothelial permeability [26], we also studied the effects of ATGL-dependent lipolysis on endothelial barrier function *in vitro*. Similarly to *in vivo* assay, OA-induced effects *in vitro* assessed by the electrical cell impedance sensor (ECIS) in terms of endothelial barrier integrity was impaired if OA was given together with atglistatin.
It is important to underline that, on the basis of functional assays *in vitro* and *ex vivo* we demonstrated the vasoprotective role of ATGL against triacylglycerol overload. As AA stored in the form of triacylglycerols pool can be re-released by the cell [18, 37], we conclude that inhibition of ATGL leading to the blockade of LDs decomposition and therefore blocking the AA release from LDs reduces the availability of AA to eicosanoid production including PGI$_2$. Indeed, our results showed an increase in the number of LDs along the inhibition of eicosanoids production in response to ATGL inhibitor after exogenous OA or AA treatment. These results highlight ATGL as the new player in PGI$_2$ generation related to LDs formation in vascular wall in response to lipid overload. Moreover, the importance of ATGL for vasoprotection may also be linked to anti-platelet, anti-inflammatory and anti-atherosclerotic activity of PGI$_2$ [46, 47].

In conclusion, in the present work using comprehensive methods including functional assays *in vivo* and *in vitro* and mechanistical studies we demonstrated that endogenous ATGL/cPLA$_2$/PGI$_2$ pathway activated by exogenous lipid overload, associated with LDs formation in endothelium and smooth muscle cells has a vasoprotective role as it counterbalances detrimental vascular effects of lipid overload and thus may play an important role in maintaining endothelial function in postprandial state. Given the key role of endothelial dysfunction occurring in postprandial phase in determining the development of atherosclerosis, our results have important pathophysiological significance that warrant further studies.

**Abbreviations**

LDs, lipid droplets; ATGL, Adipose Triglyceride Lipase; OA oleic acid; AA, arachidonic acid; AACOCF3, arachidonyl trifluoromethyl ketone; sc-560, 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazolecyclooxygenase, DuP-697, 5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl)-thiophene; cPLA$_2$, cytosolic phospholipase A2; PGI$_2$, prostacyclin, 6-keto-PGF$_{1\alpha}$, 6-Keto-prostaglandin F1$\alpha$, PGD$_2$, Prostaglandin D2; PGE$_2$, Prostaglandin E2; PGF$_{2\alpha}$, prostaglandin F2$\alpha$

**Declarations**

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**Conflict of interest**

Not applicable

**Author contributions**

Conceived and designed the study: MgS and SC. Performed the study: MgS, MS, AB, MZP, KWL, NCh, TM, BM, ICC; Analyzed the data: MgS, MS, TB, AK, AB, AM. Drafted the manuscript: MgS and SC. All authors
corrected and approved the final version of the manuscript.

**Availability of data and materials**

Not applicable

**Ethical approval**

All experimental procedures involving animals were conducted according to the Guidelines for Animal Care and Treatment of the European Communities and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All procedures were approved by the Local Ethical Committee on Animal Experiments.

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**Consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Figures
Figure 1

Lipid droplets formation in human aortic endothelial cells (HAEC) induced by exogenous AA; ATGL- and cPLA₂-dependent eicosanoid release from endogenous AA.

Effect of inhibition of atglistatin and AACOCF3 on lipid droplets formation (A) and eicosanoids release (B-E) in HAEC 4 hrs and 24 hrs after deuterated arachidonic acid (d8AA, 25 µM) addition in the presence or
absence of atglistatin (50 µM) and AACOCF3 (10 µM). Data represent mean ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple comparisons test (* p = 0.05, **** p = 0.0001).

Figure 2

Synthesis of PGI₂ from endogenous AA by cPLA₂/COX1/COX2 pathway in response to exogenous AAd8 in human aortic endothelial cells (HAEC)

The content of exogenous deuterated arachidonic acid (AAd8, 25uM) (A), endogenous AA (B) in lipid extracts and the concentration of 6-keto-PGF₁α (C) in medium +/- cPLA₂, COX1, COX2 inhibitors (AACOCF3, SC-560, DuP697) after 4 hrs and 24 hrs of HAEC incubation with AAd8. Data represent mean ± SD of three independent experiments. Statistical analysis was performed using nonparametric
Kruskal–Wallis test followed by Dunn's multiple comparisons test (* p = 0.05, ** p = 0.01, *** p = 0.001, **** p = 0.0001).

Figure 3

Lipid droplets formation in human aortic endothelial cells (HAEC) and vascular smooth muscle cells (MOVAS) induced by exogenous OA; parallel formation of PGI\(_2\) from endogenous AA in vitro.

Time course of changes in lipid droplets formation (A,B) and 6-keto-PGF\(_{1\alpha}\) release (C,D) stimulated by deuterated OA (OAd34, 100μM, 1 hr, 2 hrs, 3 hrs, 6 hrs, 24 hrs). Data represent mean ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's or Bonferroni's multiple comparisons test (* p = 0.05, ** p = 0.01, **** p = 0.0001).
Figure 4

Lipid droplets formation in isolated murine aorta in response to exogenous OA; the presence of endogenous AA.

Time course of changes in lipid droplets formation in endothelium (A) and smooth muscle cells (B) within isolated murine aorta stimulated by deuterated oleic acid (OAd34, 1mM; 1hr, 2 hrs,3 hrs, 6 hrs,24 hrs). The data are presented as the median with interquartile range (n = 4). Statistics: Kruskal–Wallis test followed by Dunn's multiple comparisons test. (* p = 0.05, ** p = 0.01).

Effect of inhibition of atglistatin and AACOCF3 on lipid droplets formation in isolated murine aorta ex vivo stimulated by OAd34 (500 µM, 24 hrs) in the presence or absence of atglistatin (50uM) and AACOCF3 (10 µM) (C). The data are presented as the median with interquartile range (n=6-8). Statistics: : Kruskal–Wallis test followed by Dunn's multiple comparisons test (* p = 0.05, ** p = 0.01, *** p = 0.001).

Representative microphotographs of immunostaining of control en face aorta and aorta treated with OA (1 mM, 24 hrs). Red, blue and green fluorescence originating from PECAM-1, cell nuclei and LDs,
respectively (D).

**Visualisation of components of murine en face aorta stimulated by OAd34 (1mM, 24 hrs) by Raman spectroscopy** (brighter colours characterizing higher intensities of respective bands). Apart from typical structures like nuclei or elastin fibres Raman imaging of en face aorta show two kinds of lipid droplets: rich in OA and without dOA (E).

**Figure 5**

**ATGL- and cPLA₂-dependent PGI₂ release from endogenous AA in isolated murine aorta in response to exogenous OA.**

Time course of changes in eicosanoids release in endothelium within isolated murine aorta stimulated by deuterated oleic acid (OAd34, 1mM; 1hr,2 hrs,3 hrs,6 hrs,24 hrs) (A-D). The data are presented as the median with interquartile range (n=6). Statistics: Kruskal–Wallis test followed by Dunn’s multiple comparisons test (* p = 0.05, ** p = 0.01, *** p = 0.001, **** p = 0.0001).

Effect of inhibition of atglistatin and AACOCF3 on eicosanoids release in isolated murine aorta *ex vivo* stimulated by OAd34 (500 µM, 24 hrs) in the presence or absence of atglistatin (50 µM) and AACOCF3 (10 µM) (E-G). The results are presented as the mean ± SD (n=6-14). Statistics: one-way ANOVA followed by Tukey’s or Bonferroni’s multiple comparisons test (* p = 0.05, ** p = 0.01, *** p = 0.001).
Figure 6

Effects of the inhibition of ATGL-dependent-lipolysis of LD induced by exogenous OA on the regulation of the endothelial barrier integrity *in vitro*

Effect of inhibition of atglistatin (50 µM) on permeability changes in HAEC 4 hrs and 24 hrs after OA administration (100 µM) measured by using ECIS method. Data represent mean ± SD of three independent experiments Statistics: one-way ANOVA followed by Tukey’s multiple comparisons test (*** p = 0.001).
Figure 7

Effects of the inhibition of ATGL-dependent lipolysis of LD induced by olive oil gavage on biochemical composition of LD and postprandial endothelial function in vivo

C57BL/6 mice were fasted for 16 hrs before olive oil administration. The dose of olive oil was chosen based on plasma triglycerides measurement with plasma TG levels peak at 360 mins (A). The results are
presented as the mean ± SD (n=4-6). Statistics: one-way ANOVA followed by Tukey’s multiple comparisons test (** p = 0.01, *** p = 0.001, **** p = 0.0001).

Raman imaging of lipid droplets formation in murine aorta isolated from mice 3 hrs, 6 hrs and 9 hrs after intra-gastric administration of the olive oil (10 ml/mg bw) (B).

Comparison of the spectrum of the olive oil with the average spectrum of endothelial LDs of an isolated blood vessel 3 hrs, 6 hrs and 9 hrs after intra-gastric administration of the olive oil (C,D) The Raman spectrum of LDs indicates higher lipid unsaturation than the administered olive oil as the ratio of the bands 1660 to 1446 cm\(^{-1}\), and 1266 to 1305 cm\(^{-1}\) is higher for the LDs spectrum than for olive oil.

**Endothelial function after olive oil administration - assessed in vivo using unique MRI-based analysis** (E). Changes in end-diastolic volume of the abdominal (A:AA) and thoracic aorta (B: TA) 30 minutes after acetylcholine administration or 30 minutes after sodium nitroprusside administration (C:AA; D:TA) in C57Bl/6 mice gavaged with olive oil (10 ml/kg bw). Measurements were placed 6 hrs after gavage of olive oil. The dose of olive oil was chosen based on plasma triglycerides measurement curve. The results are presented as the mean ± SD (n=6-14). Statistics: one-way ANOVA followed by Tukey’s multiple comparisons test (* p = 0.05, *** p = 0.001).

**Supplementary Files**

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